A High Content Primary Screening Assay determining ERK and JNK Activation

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Abstract

Here we present a rapid method for determining ERK and JNK activation, demonstrated using time course, concentration-dependence data and the effect of the MEK1/2 inhibitor UO126 on ERK activation obtained using the Acumen Explorer™ laser scanning cytometer analysing 384 well plates

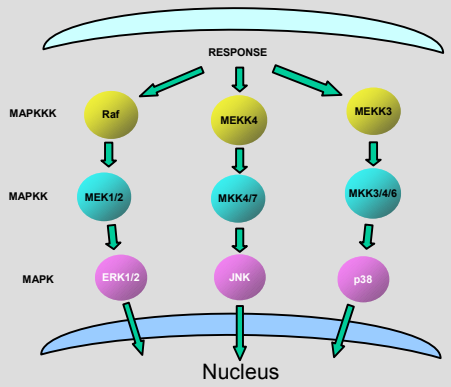
Introduction

Mitogen-activated protein kinases (MAPKs) are a widely conserved family of serine/threonine protein kinases. They consist of a three tier protein kinase cascade that acts via a family of small GTPases, which phosphorylate and activate a MAPKKK, which in turn activates the MAPKK, and in turn the MAPK. MAPK family members include the extracellular signal-regulated kinases (ERKs) and c-Jun N-terminal kinases (JNKs). The ERK pathway is stimulated by diverse extracellular signals and mediate a variety of cellular responses, including mitogenesis and differentiation (1-4) and are activated by a dual phosphorylation of threonine and tyrosine (202 and 204) on a TEY motif by the upstream kinase MEK (5,6). JNKs activation has been implicated in the immune response, oncogenic transformation and apoptosis and is activated by a dual phosphorylation on a TPY motif by the upstream kinase MKK4 (7,8).

HCS Assays

Using whole cell analysis in a primary screen enables the researcher to determine the effect of compounds in the presence of all the associated proteins in the correct stoichiometric ratio and location within the kinase cascade. Only in a whole cell environment will a multi-component pathway be present and hence assays to determine kinase activation in such a system takes account of these factors. This approach is therefore applicable in high information screening approaches to kinase target discovery.

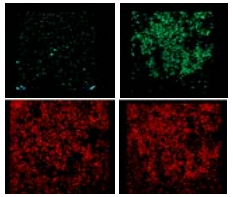
MAP Kinase Cascade



1 Assay Protocol

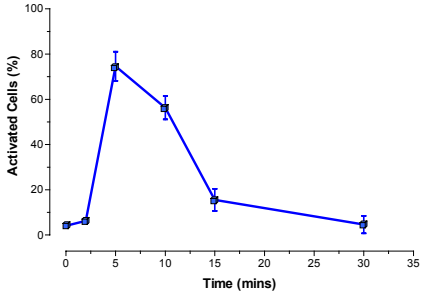
CHO cells were routinely passaged and plated out at 2,000 cells per well into a 384 well plate. Cells were serum starved overnight (for ERK assays) prior to addition of FCS or anisomycin. The reaction was terminated with 2% paraformaldehyde for 45 minutes, followed by incubation with 100% MeOH. Following blocking by 1% BSA in PBS for 1 hour at room temperature, the cells were incubated with a 1/250 dilution (in 0.1% BSA in PBS) of phospho-p44/42 MAP kinase antibody (CST #9101) or 1/50 dilution (in 0.1% BSA in PBS) of phospho-p54 JNK antibody (CST #9251) overnight at 4°C. Cells were washed 3 × 10 minutes then incubated with 1/1000 (in 0.1% BSA in PBS) goat anti-rabbit FITC antibody (Jackson; 111-096-045) for 1 hour at room temperature. Cells were washed 3 × 10 minutes and incubated with 1 µM Syto64® (Molecular Probes; S-11346) for 15 minutes. The plate was then scanned on the Acumen Explorer.

2 Acumen Explorer Well View

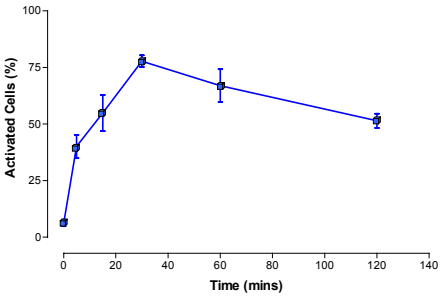


ERK activation: View of control well (left) and treated well (right) Upper panel FITC only showing active cells; lower panel Syto64® showing total cell number.

3 Time dependence curve of ERK and JNK activation in CHO cells

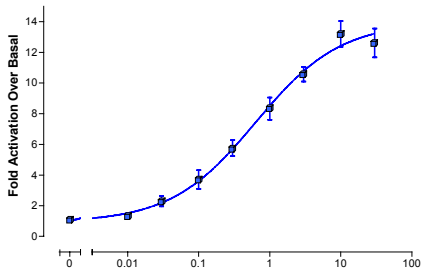


ERK activation by 10% FCS. Data represent means ± S.D. of 4 replicates and are representative of results obtained from 3 separate experiments.



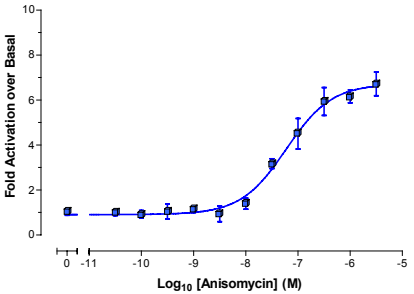
JNK activation by 10 µM Anisomysin. Data represent means ± S.D. of 4 replicates and are representative of results obtained from 3 separate experiments.

4 Concentration-dependence curve of FCS-stimulated ERK activation



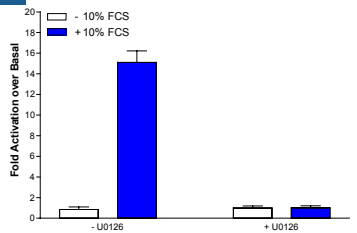
Concentration-dependence curve: EC_{50} = 0.72 mg/mL (data represent means ± S.D. of 8 replicates and are representative of results obtained from 4 separate experiments).

5 Concentration-dependence curve of anisomycin-stimulated JNK activation



Concentration-dependence curve: EC_{50} = 449 nM (data represent means ± S.D. of 4 replicates and are representative of results obtained from 3 separate experiments).

6 U0126 inhibition of ERK in CHO cells



CHO cells were incubated for 30 minutes with 10 µM of the MEK1/2 inhibitor, UO126 or DMSO as a control, prior to stimulation for 5 minutes with 10% FBS. The results demonstrate that the MEK1/2 inhibitor completely inhibits FCS-induced ERK action in CHO cells (data represent means ± S.E.M of 3 separate experiments).

Conclusion

We have shown that the Acumen Explorer can be used to quantify ERK and JNK activation in a HTS format. By scanning for 2 colours simultaneously, the total number of cells can be determined, and at the same time the number of activated cells recorded. This allows for the percentage of cells that are positive for activated kinase to be calculated. This simultaneous detection of total cell number and activated cells can be run at plate read times of as short as 5 minutes per 384 well plate and therefore allows for the assay to be used as a primary screen in the determination of activated kinases.

References

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